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MicroRNA-26a Is Strongly Downregulated in Melanoma and Induces Cell Death through Repression of Silencer of Death Domains (SODD)

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Melanoma is an aggressive cancer that metastasizes rapidly and is refractory to conventional chemotherapies. Identifying microRNAs (miRNAs) that are responsible for this pathogenesis is therefore a promising means of developing new therapies. We identified miR-26a through microarray and quantitative reverse-transcription–PCR (qRT-PCR) experiments as an miRNA that is strongly downregulated in melanoma cell lines as compared with primary melanocytes. Treatment of cell lines with miR-26a mimic caused significant and rapid cell death compared with a negative control in most melanoma cell lines tested. In surveying targets of miR-26a, we found that protein levels of SMAD1 (mothers against decapentaplegic homolog 1) and BAG-4/SODD were strongly decreased in sensitive cells treated with miR-26a mimic as compared with the control. The luciferase reporter assays further demonstrated that miR-26a can repress gene expression through the binding site in the 3′ untranslated region (3′UTR) of SODD (silencer of death domains). Knockdown of these proteins with small interfering RNA (siRNA) showed that SODD has an important role in protecting melanoma cells from apoptosis in most cell lines sensitive to miR-26a, whereas SMAD1 may have a minor role. Furthermore, transfecting cells with a miR-26a inhibitor increased SODD expression. Our findings indicate that miR-26a replacement is a potential therapeutic strategy for metastatic melanoma, and that SODD, in particular, is a potentially useful therapeutic target.

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INTRODUCTION

Metastatic melanoma is a devastating disease that is notoriously resistant to traditional chemotherapies. Recent advances in the use of BRAF inhibitors have marked the first serious progress in treating malignant melanoma in nearly 30 years (Buzaid, 2004; Cummins *et al.*, 2006; Gogas *et al.*, 2007). Yet, in spite of the success of these new drugs, they are only appropriate in roughly one-half of patients with melanomas harboring BRAF V600E mutations, and relapse and resistance are inevitable (Flaherty, 2010; Ades and Metzger-Filho, 2012). Thus, there is still a pressing need for therapies using new approaches.

Dysregulation of important cell proliferation pathways and tumor suppressors is a necessary step in oncogenesis. Recently, microRNAs (miRNA), short RNAs that target expressed mRNA for degradation, have gained much attention for the role they have in affecting protein expression in cancer tissues (Esteller, 2011; Kasinski and Slack, 2011; Sandhu and Garzon, 2011). Release 18 of miRBase (www.mirbase.org) has nearly 1,900 unique mature miRNAs annotated for the human genome (Kozomara and Griffiths-Jones, 2011), and 60% of all genes may be regulated by miRNAs (Friedman *et al.*, 2009). Thus, miRNAs represent an important regulatory layer that can affect gene expression above and beyond other cellular mechanisms. The small size and natural occurrence of miRNAs have made them attractive for use in therapy. New therapies are currently being developed based primarily on miRNA replacement, as miRNAs are most commonly downregulated in cancers and are thought to act as tumor suppressors (Kumar *et al.*, 2007). In addition, miRNA studies can be useful in identifying potential therapeutic targets that may act upstream or downstream of miRNAs.

In this study, we identified miR-26a in a microarray screen and subsequent quantitative reverse-transcription–PCR (qRT-PCR) validation as being strongly downregulated in melanoma

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Abbreviations: miRNA, microRNA; qRT-PCR, quantitative reverse-transcription–PCR; siRNA, small interfering RNA; SMAD1, mothers against decapentaplegic homolog 1; SODD, silencer of death domains; TNF, tumor necrosis factor; 3′UTR, 3′ untranslated region

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cells compared with primary melanocytes. In surveying possible targets of miR-26a, we found two proteins, SMAD1 (mothers against decapentaplegic homolog 1) and BAG family molecular chaperone regulator 4 (BAG4, also known as silencer of death domains (SODD), hereafter referred to only as SODD), that were strongly downregulated upon miR-26a treatment. SODD and to a lesser extent SMAD1 appear to be necessary to prevent apoptosis in multiple melanoma lines. We discuss the potential for melanoma therapy through miR-26a replacement or direct targeting of these proteins.

RESULTS

Microarray analysis and qRT-PCR identified miR-26a as strongly downregulated in melanomas compared with melanocytes

We performed microarray analysis to identify miRNAs that are significantly changed in human melanoma cell lines compared with melanocytes. We found a number of miRNAs that were either downregulated or upregulated (Table 1). We then performed qRT-PCR of samples from numerous melanoma cell lines and two different primary melanocyte cultures at different passages under different culture conditions (Figure 1 and data not shown). Results indicated that miR-26a was strongly downregulated in all of the melanoma cell lines tested compared with the melanocytes. Although levels of miR-26a varied considerably among both melanomas and melanocytes, the levels were strikingly lower in melanomas, from 4-fold to as much as 50-fold (average of 17.7-fold) lower than in melanocytes (Figure 1). These results clearly demonstrate that miR-26a is strongly downregulated in melanoma cells, validating our microarray results. As miR-26a showed the strongest and most consistent difference among the miRNAs that we tested (Figure 1 and data not shown), in this study, we focused on miR-26a.

Table 1. MicroRNAs (miRNAs) identified as down- or upregulated in melanomas compared with normal melanocytes by rank product statistical analysis

	Fold expression (melanoma/melanocyte)
Downregulated in melanoma	
hsa|let-7e	0.5
hsa|miR-1234	0.2
hsa|miR-125a-5p	0.3
hsa|miR-130a	0.5
hsa|miR-20b	0.7
hsa|miR-26a	0.6
hsa|miR-30a	0.6
hsa|miR-361-5p	0.6
hsa|miR-363	0.1
hsa|miR-494	0.4
hsa|miR-508-3p	0.5
hsa|miR-509-3-5p	0.3
hsa|miR-513a-5p	0.2
hsa|miR-923	0.5
Upregulated in melanoma	
hsa|miR-100	2.0
hsa|miR-222	2.7
hsa|miR-23b	2.1
hsa|miR-27b	2.4
hsa|miR-31	2.5
hsa|miR-99a	2.4

Transfection with miR-26a mimic decreases viability and increases apoptosis in multiple melanoma cell lines

To test whether low levels of miR-26a facilitate melanoma cell viability, we transfected multiple melanoma cell lines with miR-26a mimic. Viability assays, as shown in Figure 2a, demonstrate massive reduction in viability starting at 72 hours and accelerating by 96 hours after transfection with miR-26a mimic compared with transfection with negative control small interfering RNA (siRNA) in most cell lines tested, but not in normal primary melanocytes HEM_NLP2. Statistical analyses indicated that, at 72 hours, the viabilities of miR-26a-transfected cells from RPMI7951, WM278, WM852c, and 1205Lu were significantly different from those of control-transfected cells ($P<0.05$). At later time points, in addition to the same melanoma cell lines above, the viability of miR-26a-transfected HT-144 was also significantly different from the controls. On the other hand, melanoma cell lines A375 and SK-MEL-28 and primary melanocytes HEM_NLP2 were resistant to miR-26a.

Annexin V assays shown in Figure 2b corroborate the viability assays and demonstrate that apoptosis is a major mechanism of reduced viability in some cell lines. Statistical analyses indicated that miR-26a significantly increased the percentage of total Annexin V+ cells in HT-144, WM852c, WM278, and 1205Lu (Figure 2b). The appearance of the cells

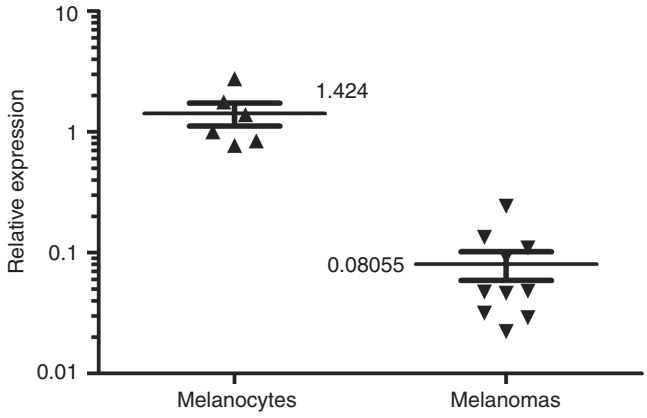

Figure 1. Scatter plot of quantitative reverse-transcription–PCR (qRT-PCR) results for miR-26a expression showing melanocytes versus melanoma cell lines. Melanocytes include two different primary melanocyte lines (HEM_NLP and HEM_NLP2) at different passages and with different culture conditions (with or without fetal bovine serum (FBS)). Melanomas include 10 different established cell lines under standard culture conditions (see Materials and Methods for a list of cell lines). All results were normalized to RNU1A as an internal standard, and denote relative expression compared with one of the melanocyte lines set at 1.0. Melanoma lines had an average of 17.7-fold less miR-26a compared with melanocytes, $P<0.0001$.

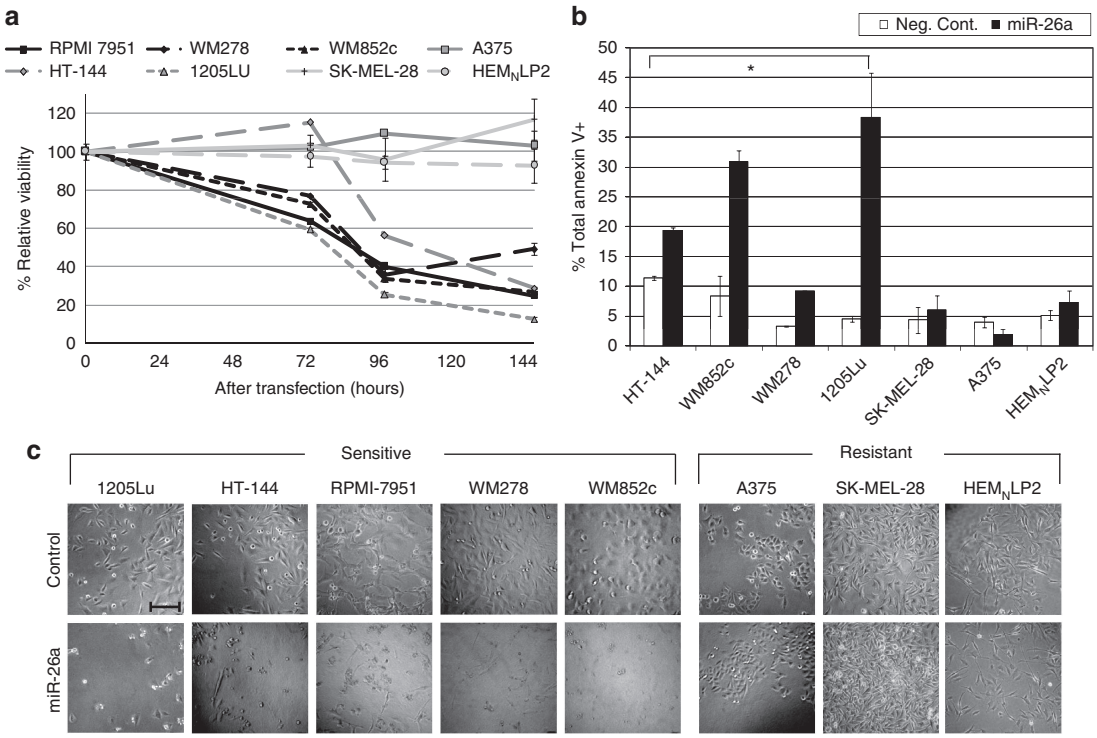

Figure 2. Treatment with miR-26a mimic induces cell death in several melanoma cell lines. (**a**) Time-course MTS assays for cells transfected with 50 nM miR-26a mimic. Results for each cell line at each time point represent the viability of cells transfected with miR-26a mimic as a percentage of those transfected with negative control. Error bars represent SEM of at least three replicates. See Results section for statistical significance. (**b**) The Annexin V assays of cells transfected with 50 nM miR-26a or negative control for 96 hours. **P*-values <0.05 for comparisons between miR-26a mimic and negative control cells. Error bars represent SEM of at least two independent experiments. (**c**) Visual appearance of cells after 96 hours of treatment. Scale bar in top left image = 0.5 mm.

(Figure 2c) is consistent with the viability and Annexin V assays, showing reduced proliferation and cell death. Cell lines, A375 and SK-MEL-28, were resistant to the effects of the miR-26a mimic, as were primary melanocytes, HEM_NLP2 (Figure 2c). Furthermore, immunoblots of lysates from cells transfected with miR-26a mimic showed that miR-26a also increased poly (ADP-ribose) polymerase cleavage in miR-26a-sensitive cells compared with those transfected with negative control siRNA (Figure 3a), further demonstrating that miR-26a induces apoptosis in these cells.

To test whether variations in transfection efficiency could account for the differences in sensitivity to miR-26a mimic, we used an identical protocol to transfect multiple melanoma cell lines and melanocytes with Allstars Negative Control siRNA conjugated with a fluorophore. Fluorescence microscopy indicated transfection efficiencies near 100%, even in cells resistant to miR-26a mimic (Supplementary Figure S1 online). In addition, we measured miR-26a in both sensitive and resistant cell lines transfected with miR-26a mimic and negative control by qRT-PCR. miR-26a levels were on the order of 10^3-fold higher in cells transfected with miR-26a compared with those transfected with negative control at 72 hours after transfection (928- and 3,866-fold higher in HT-144 and SK-MEL-28 cells, respectively). We thus concluded that differences in transfection efficiency or miRNA stability were not responsible for differences in sensitivity to miR-26a.

SMAD1 and SODD protein levels are decreased in miR-26a mimic-treated cells, inhibition of miR-26a increases SODD levels, and luciferase reporter assays confirm that SODD is a target of miR-26a

Melanoma cells treated with miR-26a mimic and negative control siRNA were collected after 72 hours and lysed for immunoblotting. We tested these lysates for a variety of possible targets to miR-26a, including EZH2, cyclins D2 and E2, metadherin, glycogen synthase kinase-3β, SMAD1, and SODD. Each of these proteins, with the exception of SODD, has been previously identified as a target of miR-26a (Luzi *et al.*, 2008; Sander *et al.*, 2008; Kota *et al.*, 2009; Mohamed *et al.*, 2010; Zhang *et al.*, 2011). SODD was identified in the TargetScan database as having a potential 3′UTR binding site for miR-26a close to the stop codon, with 99 percentile context score, in addition to two other binding sites, rendering it a strong candidate target. We found sharp reductions in the levels of SMAD1 and SODD in most sensitive cell lines, but not in the other putative targets (Figure 3a and data not shown). Resistant cell lines, A375 and SK-MEL-28, showed little or no reductions in these proteins. All sensitive cell lines showed reduction in at least one of these two proteins of $\geqslant 50\%$, although 1205Lu showed no noticeable reduction in SODD after miR-26a treatment.

To further test the role of miR-26a in targeting SODD, a putative and previously unreported target, we transfected

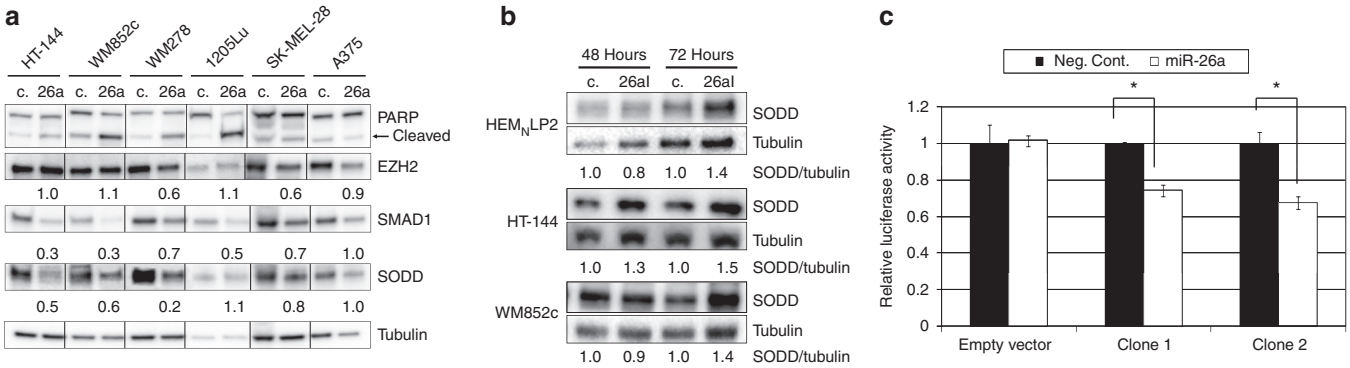

Figure 3. SODD (silencer of death domains) is a target of miR-26a. (**a**) Immunoblot. Cells were transfected with miR-26a mimic (26a) or negative control (c.) for 72 hours. Quantifications are the ratios of the proteins to tubulin with controls set to 1.0. SMAD1, mothers against decapentaplegic homolog 1. (**b**) Immunoblot. Cells were transfected with 50 nM miR-26a inhibitor (26al) or miScript inhibitor negative control (c.). (**c**) Dual-luciferase reporter assay. Luciferase reporter vectors containing SODD 3′ untranslated region (3′UTR; clones 1 and 2) or empty vectors were used. Relative luciferase activity was significantly decreased with miR-26a cotransfection (0.74 and 0.68, $P=0.002$ and 0.0013, respectively) compared with control mimic for the constructs containing the SODD 3′UTR, but not for the empty vector (1.02, $P=0.82$). Error bars represent SD of three replicates. *P-value<0.05. PARP, poly (ADP-ribose) polymerase.

primary melanocytes, HEM_NLP2, and two melanoma cell lines that are sensitive to miR-26a, HT-144, and WM852c, with an miRNA inhibitor specific to miR-26a. Lysates of these cells were compared with those transfected with a negative control inhibitor by immunoblot. We found noticeably increased levels of SODD in cells treated with the miR-26a inhibitor at 72 hours (Figure 3b), indicating that endogenous miR-26a has a repressive effect on SODD.

To further confirm the role of miR-26a in regulating SODD, we constructed a dual luciferase reporter system with 479 base pairs (bp) of the 568 bp SODD 3′UTR from the complementary DNA of a melanoma cell line containing the miR-26a binding site downstream of the *Renilla* luciferase gene (Figure 3c). We then cotransfected HEK293 cells with both miRNA mimics (miR-26a or negative control) and reporter constructs (two different clones of the reporter plasmids containing SODD 3′UTR or empty vector without any 3′UTR). The dual luciferase assays showed that at 48 hours after transfection, miR-26a significantly decreased relative luciferase activity compared with the negative control for both clones with the construct containing the SODD 3′UTR (0.74 and 0.68, $P=0.002$ and 0.0013, respectively; Figure 3c). In contrast, there was no significant difference in relative luciferase activity for the empty vector between miR-26a and control cotransfections (1.02, $P=0.82$). In addition, we also constructed reporter plasmids containing a shorter SODD 3′UTR with the same miR-26a binding site, wild-type or mutated (Supplementary Figure S2 online). The luciferase assays showed that 48 hours after transfection, miR-26a significantly decreased the relative *Renilla* luciferase activity when cotransfected with the construct containing the wild-type binding site instead of the mutant binding site (0.52, $P<0.0001$; Supplementary Figure S2 online). In contrast, mutations in the miR-26a binding site of the 3′UTR in the reporter plasmid markedly decreased relative miR-26a repression of luciferase activity (0.88 vs. 0.52, $P=0.0014$; Supplementary Figure S2 online). These results demonstrated that miR-26a can repress gene expression through the miR-26a binding site in the SODD 3′UTR.

Knockdown of SODD, and to a lesser extent of SMAD1, induces cell death in melanoma cell lines sensitive to miR-26a

To test whether miR-26a targeting of SMAD1 and/or SODD was responsible for the cell death observed upon treatment with miR-26a mimic, we knocked down these transcripts in multiple melanoma cell lines using siRNA. Cell lines were treated with up to 25 nM of total siRNAs against SMAD1 (siSMAD1) or SODD (siSODD), or both together for 120 hours and subjected to MTS assays. Cell lines WM278, HT-144, and WM852c all showed significant reduction in viability after treatment with siSODD and for the combination, and HT-144 and WM278 showed a slight reduction in viability with siSMAD1 treatment compared with negative control siRNA (Figure 4a; for concentration-dependent results, see Supplementary Figure S3 online). Cell lines 1205Lu and SK-MEL-28 showed no reduction in viability, whereas A375 showed only a slight reduction in viability with siSODD treatment compared with the control (Figure 4a). Similar results were found for the other two siRNAs (not shown). Annexin V assays after 120 hours of siRNA treatments for cell lines HT-144, WM852c, and WM278 demonstrated a significant apoptotic population ranging from 17 to 26% upon siSODD treatment, and minor apoptosis (~2–8%) for siSMAD1 treatment (Figure 4b) above the control. 1205Lu showed only slight apoptosis (~5%) with siSODD treatment. Cell lines SK-MEL-28 and A375 were essentially unaffected by siRNA treatments, according to the Annexin V assays. The appearance of the cells (Figure 4c) also indicated cell death for lines sensitive to siSODD and was similar to that with miR-26a treatment. Western blotting showed strong knockdown of the respective proteins in all cell lines treated with siSMAD1 and siSODD, except for SK-MEL-28, which had only slight knockdown (Figure 4d). Results from both MTS and Annexin V assays upon siSODD treatment are broadly consistent with results for miR-26a treatments, although there is the major exception of cell line 1205Lu. Treatment with siSMAD was similarly consistent with miR-26a results, though its effects were very small.

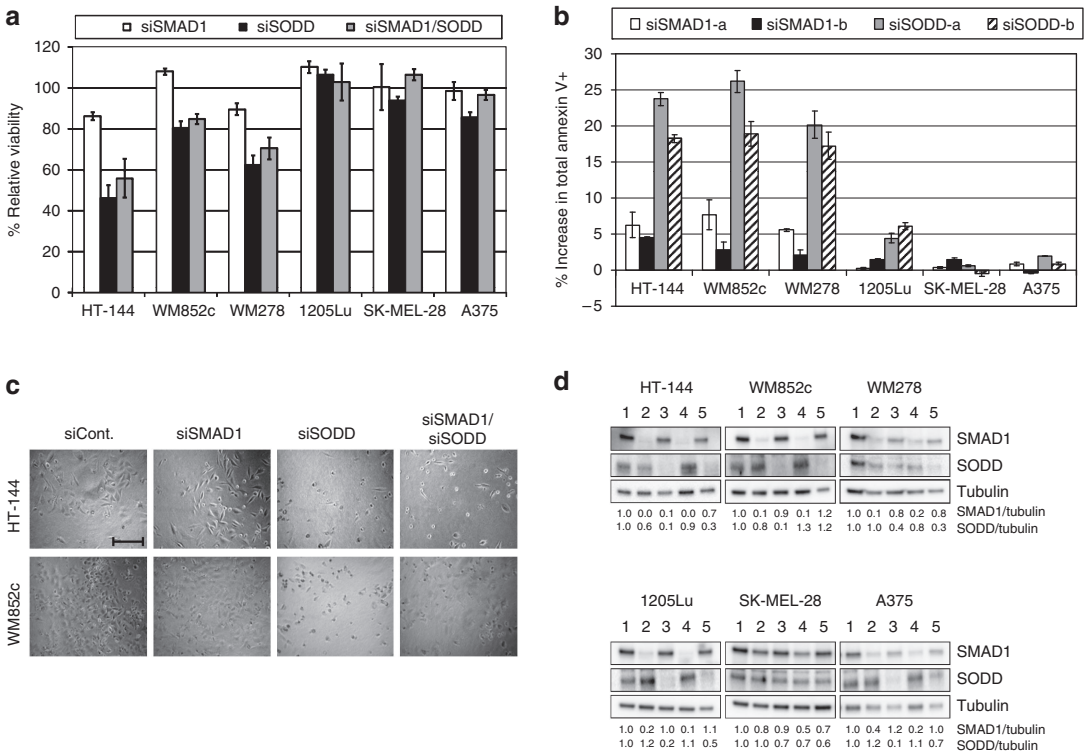

Figure 4. The effects of knocking down SODD (silencer of death domains). (**a**) MTS assays after 120 hours of treatment with 25 nM total small interferring RNA (siRNA) against SMAD1 (siSMAD1-a or siSMAD1-b), or SODD (siSODD-a or siSODD-b) normalized to the control siRNA. Shown are results using siSMAD1-a and siSODD-a; similar results were found for the other two siRNAs (not shown). Error bars represent SEM of three replicates. SMAD1, mothers against decapentaplegic homolog 1. (**b**) The Annexin V assay results after 120 hours of treatment with 25 nM siRNA normalized to the control. The *y* axis represents the increase in the percentage of total Annexin V–positive cells compared with negative control–transfected cells. Error bars represent SEM of at least two independent experiments. (**c**) Visual appearance of cells from (**a**). Scale bar = 0.5 mm. (**d**) Immunoblots of siRNA-treated-cells from (**b**). Lane 1, control; lanes 2/4, siSMAD1 (siSMAD1-a and siSMAD1-b); lanes 3/5, siSODD (siSODD-a and siSODD-b).

DISCUSSION

We identified miR-26a in a microarray screen and subsequent qRT-PCR validation as being strongly downregulated in melanoma cells compared with primary melanocytes. Prior studies of miRNAs in melanoma have identified a number of miRNAs that are involved in melanoma progression (Howell *et al.*, 2010; Mueller and Bosserhoff, 2010; Bell and Levy, 2011; Bonazzi *et al.*, 2012). These include two miRNAs that our analysis identified as upregulated, miR-27b and miR-222. However, to the best of our knowledge, none of the miRNAs that we identified as downregulated have been previously implicated in melanoma, although several miRNAs, in addition to miR-26a, such as miR-let7e, miR-494, and miR-30a, are known to be downregulated in other cancers (Mitra *et al.*, 2011; Olaru *et al.*, 2011; Kumarswamy *et al.*, 2012).

Downregulation or polymorphisms of miR-26a have previously been implicated in a variety of cancers, including nasopharyngeal carcinoma (Lu *et al.*, 2011), breast cancer (Zhang *et al.*, 2011), hepatocellular carcinoma (Kota *et al.*, 2009), oral cancer (Clague *et al.*, 2010), and colon cancer (Boni *et al.*, 2011). Kota *et al.* (2009) have successfully demonstrated miR-26a replacement therapy in a mouse model of hepatocellular carcinoma. In addition, downregulation of miR-26a appears to have roles in other diseases (Leeper *et al.*, 2011).

We found that treatment of melanoma cell lines with a miR-26a mimic induced substantial cell death in multiple melanoma lines, but not in primary melanocytes, indicating that reduction of miR-26a is necessary for the survival of most melanomas. However, we found melanoma cell lines resistant to the mimic, including A375 and SK-MEL-28, showing that in at least some melanomas, compensatory mechanisms must exist and/or there is incomplete degradation of miR-26a targets. Experiments with fluorescently labeled siRNA demonstrated that transfection efficiency with our protocol was extremely high, and qRT-PCR experiments showed high miR-26a levels in cells transfected with miR-26a mimic, including resistant cells. Therefore, it is highly unlikely that resistance was the result of insufficient miR-26a expression. Among the sensitive cell lines was RPMI7951, which is a p53-null line (Reuland *et al.*, 2011), indicating that the mechanism of cell death is not p53 dependent.

We searched for putative targets of miR-26a by first testing targets previously reported in other studies. EZH2, for example, is commonly cited as an important target of miR-26a (Sander *et al.*, 2008; Wong and Tellam, 2008; Alajez *et al.*, 2010; Zhang *et al.*, 2011). Although we found some reduction

of EZH2 in some miR-26a-treated cells, the reduction was low and inconsistent (Figure 3a). In addition, we treated cell lines with the histone methylation inhibitor DZNep, which depletes EZH2 (Tan *et al.*, 2007), and found that it had little effect even at high doses (Supplementary Figure S4 online), with the paradoxical exception of cell line A375 that, although sensitive to DZNep, is resistant to miR-26a treatment. We therefore concluded that miR-26a was not killing melanoma cells through EZH2 knockdown.

Other previously validated targets of miR-26a include glycogen synthase kinase-β (Mohamed *et al.*, 2010), cyclins D2 and E2 (Kota *et al.*, 2009; Lu *et al.*, 2011; Zhu *et al.*, 2012), metadherin (Zhang *et al.*, 2011), and SMAD1 (Luzi *et al.*, 2008; Nigam *et al.*, 2010). Of these, only SMAD1, a transcription factor involved in bone morphogenic protein signaling, showed clear and consistent repression by miR-26a in the melanoma cell lines we tested (Figure 3a and data not shown). We also queried the TargetScan database to identify other potential targets of miR-26a. A binding site in the 3′UTR of SODD was predicted with a 99 percentile context score, and is also close to the stop codon (position 130–137). In addition, SODD encodes a member of the BAG1 family, a potential antiapoptotic protein (Antoku *et al.*, 2001). As with SMAD1, we found that miR-26a caused strong and consistent downregulation of SODD protein. We also found that the inhibition of endogenous miR-26a in normal melanocytes and two melanoma cell lines leads to increased SODD levels (Figure 3b), and we further found that miR-26a can repress the gene expression through the native SODD 3′UTR using luciferase reporter assays (Figure 3c and Supplementary Figure S3 online). Taken together, the evidence indicates that SODD is a target of miR-26a in melanoma cells.

To test the possible involvement of SMAD1 and/or SODD in melanoma cell death induced by miR-26a replacement, we specifically knocked down these two proteins with siRNA. The results indicated that SODD expression, at least, is necessary for preventing cell death in multiple melanoma lines. We found only minor effects by knocking down SMAD1 alone, although the possibility remains that this protein has crucial roles under different contexts. Interestingly, one cell line that was highly sensitive to miR-26a mimic, 1205Lu, was completely insensitive to siSODD and siSMAD1 treatments (compare Figures 2 and 4). This was likely not because of insufficient knockdown (Figure 4d), but rather implies that there are additional targets of miR-26a that account for the sensitivity of 1205Lu to the mimic. It is well known that miRNAs have many targets and that these vary according to the cell type in a context-dependent manner (Didiano and Hobert, 2006). It is not clear to us why this cell line is highly sensitive to the miRNA mimic yet not affected by the knockdown of SODD or SMAD1. We hypothesize that one or more targets, other than SODD or SMAD1, mediate the effects of miR-26a in the 1205Lu cell line. The identification of these additional targets and the potentially complex mechanism of their interplay will require further study. Similarly, the resistance of two melanoma cell lines to both miR-26a and the siRNAs against SMAD1 and SODD would seem to indicate that these proteins are essential only in a subset of melanoma cells. Significant knockdown of both proteins was achieved in siRNA-treated A375 cells (Figure 4d), and yet this had no effect on viability. Taken together, it appears that although SODD is a potentially critical target of miR-26a in many melanomas, its knockdown by miR-26a treatment can only cause cell death in a subset of melanoma cell lines.

To the best of our knowledge, SODD has not been previously reported as a target of miR-26a and as an antiapoptotic protein in melanoma. SODD is known as an inhibitor of the death domains of the tumor necrosis factor (TNF) receptor 1, preventing trimerization of the receptor subunits in the absence of specific signaling (Jiang *et al.*, 1999; Miki and Eddy, 2002). Upon TNF-α binding, the subunits of the receptor trimerize and SODD is quickly released. Activated TNF receptors can then lead to either apoptosis or NF-κB activation. In addition, SODD is known to interact with Hsp70, death receptor 3, and the antiapoptotic protein Bcl-2 (Jiang *et al.*, 1999; Antoku *et al.*, 2001; Brockmann *et al.*, 2004). SODD is overexpressed in pancreatic cancer and leads to resistance of TNF-α-induced cell death (Ozawa *et al.*, 2000), and increased SODD is correlated with the severity of acute lymphoblastic leukemia in children, and the downregulation of SODD and NF-κB induces apoptosis (Tao *et al.*, 2007). As the balance between pro- and anti-apoptotic signaling in TNF receptor 1 activation is critical in melanoma (Ivanov *et al.*, 2003), it is likely that SODD acts to tip the balance away from cell death and toward NF-κB activation, which is constitutively active in melanoma and important in its progression (Poser and Bosserhoff, 2004; Madonna *et al.*, 2012). In addition, SODD could have a role in the antiapoptotic functions of Bcl-2 family members, as we and others have found that these proteins are critical mediators of cell survival in melanoma (Reuland *et al.*, 2011, 2012). Further study is needed to test these and other hypotheses, but regardless, our data indicate that SODD is essential for cell survival of a subgroup of melanoma cells without any other triggers, suggesting that SODD is a potential target for treatment of certain types of melanoma.

In conclusion, we have found that miR-26a is strongly downregulated in melanoma cells compared with normal melanocytes, and that replacement of miR-26a induces cell death in multiple cell lines. We have identified the antiapoptotic protein SODD as a target of miR-26a, and found that specific knockdown of SODD produces cell death in some, but not all, cell lines sensitive to miR-26a. Although the mechanistic details of its function in melanoma require more study, SODD is a potential target for melanoma therapy. In addition, our data imply that miR-26a must act on additional targets, and that miR-26a replacement therapy is a promising strategy for treating melanoma.

MATERIALS AND METHODS

miRNA microarrays

MicroRNAs from melanoma cell lines SK-MEL-28 and HT-144 were compared with those from two normal melanocyte cultures. Each sample was run in triplicate for the experiment. Total RNAs were extracted using an miRNeasy kit (Qiagen, Valencia, CA), and then enriched for small RNAs using an RNeasy MinElute kit (Qiagen)

before being sent to the University of Colorado Cancer Center Microarray Core for miRNA microarray analysis. RNA samples were analyzed on a Bioanalyzer Small RNA chip for quality control, then labeled and run on CombiMatrix human miRNA arrays (CombiMatrix Diagnostics, Irvine, CA) by the Core, as directed by the manufacturer. For data normalization, the CombiMatrix recommended method was used: in brief, background signaling was estimated as the lowest 5% of all signaling, including perfect matches and the mismatch controls, and data were then normalized from the arrays using global scale factors to bring all backgrounds to the same value. For filtering, we removed any miRNA with no normalized signals >200, or miRNAs with an intensity $<1{,}000$ in all samples, and only kept miRNAs with signaling on perfect matches that exceeded background by at least $2\times$, and perfect match values that exceeded $1.2\times$ (the intensity of the double mismatch control). We then used Rank Product statistical analysis as reported by Breitling *et al.* (2004) to identify up- or down-regulated miRNAs in melanoma compared with normal melanocytes. The Rank Product analysis is a simple nonparametric statistical method based on ranks of fold changes to detect differentially expressed genes in replicated array experiments (Breitling *et al.*, 2004). The rank products were calculated using the same method and procedure with Excel as described by Breitling *et al.* (2004).

Plasmid constructs, transfections, and luciferase reporter assays

TargetScan (http://www.targetscan.org/) was used to identify potential miR-26a binding sites in the SODD 3′UTR (called BAG4 in TargetScan). Total RNA from melanoma cell line WM852c was used to generate complementary DNAs and to clone the 3′UTR of SODD (NM_001204878). The SODD 3′UTR is predicted to be a 568-bp fragment (see http://www.origene.com/MicroRNA/3-UTR-Clone/SC207066.aspx) or a ~2.8-kb fragment (in TargetScan). We attempted to amplify both the 568 bp and ~2.8 kb 3′UTR from complementary DNAs of multiple melanoma cells; however, we could only amplify the 568-bp 3′UTR. It is possible that melanoma cells mainly have the SODD transcript with the 568-bp 3′UTR. Thus, we cloned 479 bp of this 568-bp 3′UTR into a pSiCheck2 reporter plasmid at *Xho*1/*Not*1 sites for luciferase activity assays, using the following PCR primers: 5′- ACTGCTCTGCTCGAGAGCCTGTTACT AACTTGAC-3′ and 5′AATTAGCGGCCGCCTGCAAATAACAAAA CAAAACAGAAGTCC-3′. The constructed psiCHECK2 plasmids were verified by DNA sequencing (Colorado Cancer Center DNA Sequencing Core). This cloned SODD 3′UTR, from complementary DNAs of melanoma cells, contains one predicted binding site for miR-26a.

HEK293 cells were cotransfected with luciferase reporter plasmids (psiCHECK2 vector with or without indicated SODD 3′UTR, 0.25 μg) and mimics of miR-26a or Allstars Negative Control siRNA (25 nM) in 24-well plates using the Lipofectamine 2000 reagent as directed by the supplier (Invitrogen, Carlsbad, CA). After 48 hours, the cells were lysed and assayed for luciferase activity using the Dual Luciferase Reporter Assay kit (Promega, Madison, WI) according to the manufacturer's protocol on a microplate reader (BioTek, Winooski, VT). The psiCHECK2 reporter construct contains both firefly and *Renilla* luciferase genes under the control of constitutive promoters, and firefly luciferase activity was used as an internal control. The relative *Renilla* luciferase activity was calculated as the ratio of *Renilla* to firefly luciferase activity for each sample, and the relative *Renilla* luciferase activities were normalized to 1.0 for negative control cotransfected cells.

Statistics

All comparisons were evaluated by two-tailed unpaired *t*-tests using the program GraphPad Prism (GraphPad Software, La Jolla, CA), with *P*-values of <0.05 considered significant.

Other methods

Further information about other materials and methods used in this work are provided in the Supplementary Material online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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